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INTRODUCTION

There is abundant evidence that increased expression of growth factors and increased activity growth factor receptors, particularly those of the fibroblast growth factor (FGF) and epidermal growth factor (EGF) families, play an important role in human prostate cancer. Recently, a new family of regulators of FGF and EGF activity has been identified. The sprouty gene was originally identified as a negative regulator of FGF and EGF receptor signaling in *Drosophila* and it has been proposed that it may carry out these two functions by interfering with the RAS signaling pathway. Four human homologues have been identified, but their role in the human prostate and prostate cancer is not well understood. Based on our own preliminary data and analysis of the Cancer Genome Anatomy and Unigene databases, sprouty-1 is the major human sprouty homologue expressed in human prostate and it is significantly downregulated in approximately 70% of extensive, clinically localized prostate cancers. We have now confirmed this on an independent set of prostate cancer RNAs (see attached manuscript). Loss of this negative regulation may lead to unrestrained signal transduction by FGF and EGF family members, which could result in increased proliferation and may enhance tumor aggressiveness and be correlated with clinical, pathological and biological parameters in human prostate cancer.

BODY

As outlined in our Statement of Work, we have sought to accomplish nine tasks in our initial two years of funding. We have accomplished or made substantial progress on all of these tasks. A manuscript describing the role of sprouty-1 in prostate cancer has been submitted to *Cancer Research*. A copy of this manuscript is attached and will be referred to below.

Task 1: Clone full length human sprouty-1 cDNA (Months 1-3)

We have cloned a full-length sprouty-1 cDNA and this has been sub-cloned into the pCEP4 expression vector as well as pAVS6 for production of adenovirus.

Task 2: Construct adenovirus expressing sprouty-1 gene (Months 3-12)

We have generated a recombinant sprouty-1 adenovirus and carried out large scale preparations of this virus. We have confirmed that this adenovirus expresses sprouty-1 protein by Western blotting with anti-sprouty-1 antibody using extracts of LNCaP cells infected with either sprouty-1 adenovirus or control green-fluorescent protein (GFP) adenovirus.

Task 3: Develop prostate cancer cell lines with high sprouty-1 expression (Months 3-12)

As described above we have cloned the full length sprouty-1 cDNA into the pCEP4 expression vector. We have performed stable transfection of the LNCaP cell line, isolated stable transfectants and evaluated for sprouty-1 expression by quantitative RT-

PCR. Analysis of the growth rate of these cell lines reveals that there is inhibition of growth relative to parental LNCaP cells (See Progress Report 2002). It should be noted that it was difficult to establish cell lines expressing sprouty-1 due to low numbers of colonies following transfection. We have evaluated the isolated clones for sprouty-1 expression by quantitative RT-PCR and they have very low expression of sprouty-1 mRNA, consistent with a strong selection against sprouty-1 expression in cancer cells. Thus even low levels of sprouty-1 expression can inhibit growth of prostate cancer cells. Other cancer cell lines, such as PC3, also show this very strong negative selection (see Fig 5, attached manuscript). Therefore, we have shifted our approach and are trying to establish stably transfected cell lines with inducible expression of sprouty-1 using the GeneSwitch system (Invitrogen). In this system the gene of interest (sprouty-1) is cloned into a vector in which expression is induced by a modified progesterone receptor when it is activated by binding mifepristone. We have made the appropriate expression constructs and we are currently trying to select prostate cancer cell lines with low basal expression of sprouty-1 that is inducible by RU486.

Task 4: Clone sprouty-1 genomic locus, identify intron-exon structure and identify promoter (Months 1-12)

We have cloned the sprouty-1 gene as a single BAC clone. We have also determined the sequence of promoter region from database. We have verified its activity as promoter by construction of plasmid with 850 bp of the putative promoter driving a Luciferase reporter gene which was then transiently transfected into NIH3T3 cells. This construct expresses Luciferase robustly in NIH3T3 cells and transcription is upregulated by exogenous FGF2. We have used this construct to demonstrate that in prostate cancer cells there is loss of transcriptional regulation by FGF2 (Figure 4, attached manuscript). Consistent with this finding, we have shown that treatment of prostate cancer cell lines with FGF2 does not lead to increased levels of sprouty-1 mRNA, in contrast to primary cultures of normal prostatic epithelial cells in which treatment with FGF2 induces a 5-fold increase in sprouty-1 mRNA (Figure 3, attached manuscript). We have concluded that in prostate cancer there is disruption of the normal regulation of sprouty-1 mRNA expression and this accounts, at least in part, for the decreased expression of sprouty-1 in prostate cancers.

Task 5: Raise and characterize rabbit polyclonal anti-sprouty-1 antisera (Months 1-12)

We have recently obtained a commercially available anti-sprouty-1 antibody and have developed conditions for using this antibody for immunohistochemical analysis of paraffin embedded prostate cancer tissues. We have also evaluated antisera from four rabbits with two different sprouty-1 peptides. The commercially available antiserum was superior to our antibodies and so we have carried out immunohistochemical studies with this antiserum.

Task 6. Immunohistochemistry of human prostate cancer tissue specimens (Months 12-24)

We have used the antiserum described above to analyze the expression of sprouty-1 protein in more than 600 clinically localized prostate cancers using tissue microarrays. These slides have been scanned and data analysis is currently underway. We have also analyzed TURPs from men with androgen independent cancer and autopsy tissues from men dying from prostate cancer. After analysis of this data further studies will be undertaken if indicated, particularly analysis of a newly fabricated array with 150 metastatic prostate cancers. These studies should allow us to make a comprehensive determination of the role of sprouty-1 expression in prostate cancer and whether loss of expression has prognostic significance.

Task 7: Evaluation of effect of sprouty-1 expression in normal and immortalized prostate epithelial cells (Months 12-18)

Biological experiments reveal that this sprouty-1 adenovirus markedly inhibits colony formation and net proliferation in LNCaP and DU145 cells (see Progress Report 2002 for details). The results of stable transfection studies (as described above and in the attached manuscript) also reveal a markedly deleterious effect of sprouty-1 expression on prostate cancer cell viability.

Task 8: Determine role of sprouty-1 in tumorigenesis (Months 12-18)

In order to proceed to our in vivo studies we will need to develop prostate cancer cell lines with inducible expression of sprouty-1 so we can see the effect in vivo of more moderate increases in sprouty-1 expression on tumorigenesis since higher level expression leads to complete loss of viability. The stable sprouty-1 expressing prostate cancer cell lines we established, while showing inhibition of growth, have low sprouty-1 expression (presumably due to selection against higher expressing clones) and are not really suitable for our planned in vivo studies. As described above we are currently establishing prostate cancer cell lines with inducible expression of sprouty-1.

Task 9: Analysis of human prostate cancers for mutation of sprouty-1 gene (Months 18-24)

We have identified the intron-exon boundaries and developed PCR primers for mutation analysis by analysis of human genome project database. Fortunately, the sprouty-1 gene consists of only two exons, facilitating mutation analysis. We have analyzed 30 human prostate cancer DNAs and have found no evidence of somatic mutation, consistent with our initial hypothesis that alteration of mRNA expression is the primary mode by which sprouty-1 activity is decreased in prostate cancer. We did find one germline polymorphism/mutation but the biological significance of this missense mutation is unclear (see attached manuscript for details).

KEY RESEARCH ACCOMPLISHMENTS

- * Cloned full-length sprouty-1 cDNA and subcloned into mammalian expression vector
- * Created replication deficient adenovirus expressing sprouty-1 and demonstrated that it markedly inhibits proliferation and colony formation by prostate cancer cells
- * Established stably transfected LNCaP cell lines that express sprouty-1 and shown that these cell lines have decreased proliferation relative to controls, even though they have relatively low expression of sprouty-1.
- * Cloned sprouty-1 genomic locus and established intron-exon structure of sprouty-1 gene
- * Performed mutation analysis of the sprouty-1 gene in 30 human prostate cancers.
- * Cloned an 850 bp promoter fragment of the sprouty-1 gene and demonstrated that it will act as an FGF regulated promoter in NIH3T3 cells.
- * Established that prostate cancer cell lines do not have appropriate induction of sprouty-1 after FGF stimulation and that this is due to loss of transcriptional regulation.
- * Developed procedures for immunohistochemical analysis of sprouty-1 expression and have begun large-scale studies of sprouty-1 expression in prostate cancer tissues.

REPORTABLE OUTCOMES

- * Manuscript submitted to *Cancer Research*

CONCLUSION

Sprouty-1 expression is downregulated in the majority of human prostate cancer that we have evaluated to date. Our studies have indicated that this is due, at least in part, to altered transcriptional regulation of the sprouty-1 gene in prostate cancer cells. The work described above reveals that sprouty-1 expression profoundly inhibits proliferation of prostate cancer cells *in vitro*. Future work will focus on:

- 1) completing a comprehensive evaluation of the expression of sprouty-1 in human prostate cancer by immunohistochemistry
- 2) evaluation of prostate cancer DNAs for methylation or homozygous deletion of the sprouty-1 gene to determine if some prostate cancers may lose sprouty-1 expression through these mechanisms
- 3) determining the effect of sprouty-1 expression on the biological behavior of prostate cancer cells *in vivo* by developing LNCaP cell lines with inducible expression of sprouty-1 which will then be used for orthotopic injection after which tumor progression will be evaluated in the presence or absence of inducer (mifepristone).

Sprouty1, an inhibitor of fibroblast growth factor signal transduction, is down-regulated in prostate cancer

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Abbreviations: fibroblast growth factor (FGF); fibroblast growth factor receptor (FGFR);
polymerase chain reaction (PCR), epidermal growth factor receptor (EGFR), mitogen-activated
protein kinase (MAPK), receptor tyrosine kinases (RTKs)

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ABSTRACT

A considerable body of evidence indicates that alterations of fibroblast growth factors (FGF) and their receptors contribute to prostate cancer progression. Recently a new family of regulators of FGF activity has been identified. The Sprouty gene family negatively regulates FGF signaling in a variety of systems and could potentially limit the biological activity of FGFs in prostate cancer. Using quantitative real-time PCR analysis, we have found that Sprouty1 is down-regulated in neoplastic prostate tissues when compared with normal and hyperplastic prostate tissues. We have also found that in prostate cancer cells that there is loss of the normal up-regulation of Sprouty1 mRNA in response to FGFs and this is due primarily to loss of transcriptional up-regulation following FGF stimulation. The decrease in Sprouty1 expression in the human prostate cancer, despite elevated levels of FGF ligands and FGF receptors, implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer.

INTRODUCTION

Prostate cancer is the most common visceral cancer in men and the second leading cause of cancer-related death. The lack of effective therapies for advanced prostate cancer reflects, in part, the lack of knowledge about the molecular mechanism involved in the development and progression of this disease ¹. Normal prostate growth is controlled by a variety of polypeptide growth factors, including members of the fibroblast growth factor (FGF) gene family ^{2,3}. A considerable body of evidence indicates that alterations of these growth factors and their receptors contribute to prostate cancer progression. Yan et al ⁴ have shown in the Dunning rat model system that as these transplantable tumors progress from a mixed stromal-epithelial phenotype to a stromal independent phenotype that there are changes in the isoforms of FGF receptors expressed, consistent with autocrine stimulation of growth. In humans, multiple FGFs have been shown to be up-regulated in prostate cancer. For example, FGF2 is significantly up-regulated in prostate cancers when compared with uninvolved prostate ⁵. Expression of FGF6 by prostate cancer cells has been identified in 40% of human prostate cancers *in vivo* ⁶, and the majority of prostate cancers overexpress FGF8 ⁷⁻⁹. In addition, increased expression of FGFR-1 is present in poorly differentiated human prostate cancers *in vivo* ^{5,10}. Autocrine expression of FGFs and expression of FGF receptors has been reported in all of the commonly used prostate cancer cell lines i.e., PC-3, DU145 and LNCaP ^{11,12} whilst prostate cancers express appropriate receptors to respond individually to these FGFs ¹³⁻¹⁵.

Recently a new family of regulators of FGF activity has been identified. Sprouty was originally identified as an antagonist of Breathless FGF receptor signaling during tracheal development in *Drosophila* ¹⁶. Subsequent studies have shown that Sprouty inhibits signaling mediated by the FGF receptor (FGFR) and the epidermal growth factor receptor (EGFR) during

eye development and oogenesis in *Drosophila*¹⁷⁻¹⁹. During *Drosophila* eye development, Sprouty seems to inhibit the activation of mitogen-activated protein kinase (MAPK) upstream of Ras function²⁰, whereas during wing development, it is reported to inhibit MAPK downstream of Ras function²¹.

Four mammalian genes have been identified with sequence similarity to *Drosophila* sprouty²². The mammalian Sprouty family members are expressed in highly restricted patterns in the embryo in early development and their expression shows a close correlation with known sites of FGF signaling²³⁻²⁵, which suggests that they may also function as negative regulators in FGF signaling during vertebrate embryonic development. All Sprouty proteins share a unique, highly conserved cysteine-rich domain at the carboxyl terminus, believed to be critical for targeting them to phosphatidylinositol (4,5-bisphosphate (PtdIns[4,5]P2) in the plasma membrane, thus allowing their inhibitory role on the MAPK pathway^{26,27}. The N-terminal portion of the Sprouty proteins is less conserved as it exhibits only 25-37% identity among the different mouse family members. These sequence difference could be responsible for the functional divergence among the Sprouty proteins. It has been shown that after growth factor stimulation, Sprouty1 and Sprouty2 translocate to the plasma membrane, become tyrosine-phosphorylated, and interact with Grb2, thus inhibiting recruitment of the Grb2-Sos complex to docking adaptor proteins such as FRS2 or Shp2²⁸. It is likely that Sprouty proteins can also act at additional stages of receptor tyrosine kinases (RTKs) signaling, since Sprouty2 has been shown to inhibit fibroblast growth factor (FGF)-mediated extracellular-signal-regulated kinase activated at the level of Raf²⁹, whereas Sprouty4 inhibits vascular endothelial growth factor receptor signaling upstream of Ras³⁰. In contrast, EGFR signaling is not reduced following

expression of Sprouty2 or Sprouty4³¹. It is thus conceivable that Sprouty proteins control RTK activation at different stages, with some additional regulatory mechanisms still unknown.

A search of the Unigene database (www.ncbi.nlm.nih.gov/UniGene) and the Cancer Genome Anatomy Project database (www.ncbi.nlm.nih.gov/ncicgap) indicates that Sprouty cDNAs are present in cDNA libraries from many human tissues including the prostate, with Sprouty1 being the most abundant human Sprouty homologue expressed in human prostate. However, the role of Sprouty1 in human prostate cancer is not known and little is known about alterations of regulatory molecules that may down-regulate growth factor signals in prostate cancer cells. An important consideration is that if Sprouty proteins can be up-regulated in prostate cancer by FGF stimulation this would tend to inhibit any effects of FGF receptor activation in the neoplastic cells, and negate the effects of the increased FGF expression in cancer tissues. To address this issue, we have investigated the expression of Sprouty1 in normal and neoplastic prostate tissues. Using quantitative real-time PCR analysis, we have analyzed the expression level of Sprouty1 in normal, hyperplastic (BPH) and neoplastic human prostate tissues. We present data to demonstrate that Sprouty1 is down-regulated in neoplastic prostate tissues when compared with normal and BPH cases. We have also found that in prostate cancer cells that there is loss of the normal up-regulation of Sprouty1 mRNA in response to FGFs and this is due primarily to loss of transcriptional up-regulation following FGF stimulation. The marked decrease in Sprouty1 expression in the human prostate cancer implies a loss of an important growth regulatory mechanism in prostate cancers that may potentiate the effects of increased FGF and FGFR expression in prostate cancer.

MATERIALS AND METHODS

Plasmid construction. Plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) was used for the expression of Sprouty1 full length cDNA. For the construction of pcDNA-Sprouty1, the coding sequence of Sprouty1 was amplified from pCMV-Sport6 plasmid containing the full length Sprouty1 cDNA (ATCC#3461395) in a PCR reaction using primers designed against the published Sprouty1 sequence as follows: Forward primer 5'- **AAGCTTAGACATGGATC**-CCCAAAATC-3' and a Reverse primer 5'-**GAATTCTGATGGTTACCCTGACCCC**-3' (The underlined sequence indicates *Hind* III and *EcoRI* sites in the Forward and Reverse primers respectively; bold and underlined shows the start and stop sites in the forward and reverse primers respectively; italicized and underlined G indicates an engineered G at position -3 to ensure proper initiation of translation). The PCR product was digested with *EcoRI* and *Hind* III and sub-cloned into pcDNA3.1. For the construction of pGL-Sprouty1, the Sprouty1 promoter region was amplified from normal prostate genomic DNA in a PCR reaction using as Forward primer 5'-**GAGATCTCCTCCATCCCCGATTAC**-3' and Reverse primer 5'-**GAGATCTGGGTTCACCGAGGGCCG**-3' (*Bgl* II restriction site is shown as underlined nucleotides) to amplify a 737 bp product. The PCR product was digested with *Bgl* II restriction endonuclease and cloned into the pGL-3 luciferase reporter plasmid (Promega, Madison, WI). Both Sprouty1 constructs were verified by restriction digestion analysis and sequencing.

Mutation analysis of the Sprouty1 coding region. Total genomic DNA was extracted from 26 prostate tissue samples (including 23 radical prostatectomies [all 70% or more cancer], 1 metastasis and 2 benign tissues from radical prostatectomies) as described previously³². The DNAs were used in standard PCR reaction conditions with 3 sets of primers to amplify the entire Sprouty1 coding region. Set 1 primers (SF1); Forward 5'-ACGAGCACAGACACACAAG-3'

and Reverse 5'-CAACCCACCTCCAAAAATCA-3'; Set 2 primers (SF2); Forward 5'-CCTTCTTGGATAGCCGTCA-3' and Reverse 5'-CCCTTCAAGTCATCCACAATC-3'; Set 3 primers (SF3); Forward 5'-AGGACCCCAGCATCATTGTA-3' and Reverse 5'-GTGGCTTGTGTCTGTGCT-3'. The nucleotide positions for the amplification products as given by the Genbank accession number (XM_036349) are 137-628, 394-843 and 605-1317. The PCR products were purified and sequenced using the respective set of primers for each product.

Preparation, quantification and dilution of DNA standards. The Sprouty1 plasmid, keratin-18 plasmid (ATCC #MGC-9348) and β -actin plasmid (ATCC #MGC-10559) were prepared using the Qiagen Maxi-prep Kit (Qiagen, Valencia, CA). Quantification of plasmid was performed spectrophotometrically. The measurements of the plasmid concentration were done in duplicate and then converted to copy number. A dilution series of each plasmid (10^9 to 10^1 copies) was used as a DNA standard for real time PCR.

Primer design and synthesis for real-time PCR. Oligonucleotide primers for Sprouty1 were Forward 5'-TGTCCGAAAAGGATTTCAGATGC-3'; and Reverse 5'-ACTGCCACTGCCA TGTTGAT-3' and were designed using the Molecular Beacon program (PREMIER Biosoft International). Oligonucleotide primers for β -actin were Forward 5'- AGCACGGCATCGTCA CCAACT-3' and Reverse 5'- TGGCTGGGTGTTGAAGGTCT-3' and for keratin 18 were Forward 5'- AGGGCTCAGATCT- TCGCAAAT-3' and Reverse 5'- GTCATCAATGA CCTTGCGGAG-3'. Primers were carefully designed to cross exon/intron regions, avoid the formation of primer-dimer, hair pin and self complementarity. Synthetic oligonucleotide primers were obtained from Sigma Genosys (The Woodlands, TX). The nucleotide positions for the amplification products as given per the Genbank accession numbers are 275-373, 458-622 and

256-435 for Sprouty1 (XM_036349), Keratin 18 (BC020982) and β -actin (BC004251), respectively.

cDNA synthesis and quantitative real-time PCR. Total RNA extracted from cells and tissues using TRIzoL Reagent (Invitrogen) was used in first strand DNA (cDNA) synthesis using Invitrogen SuperScriptTM first strand synthesis system for RT-PCR and according to the manufacturer's protocol. Real-time PCR was carried out by adding 5 μ l of the template cDNA to a final 25 μ l reaction volume containing (3mM MgCl₂, 0.4 μ M each forward and reverse primers and 2.5 μ l of LC-FastStart DNA Master SYBR GREEN 1 (10X; Roche, Indianapolis, IN)). Real-time PCR was done using the iCycler iQ instrument (Bio-Rad Laboratories, Hercules, CA) and incorporating the following optimized PCR reaction conditions: The amplification of Sprouty1 was carried out as follows: a 3 minute hot start at 95°C followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing at 61°C for 30 seconds. The amplification protocol for β -actin and keratin 18 was carried out as follows: a 3 minute hot start at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 20 seconds and a 72°C extension for 30 seconds. Each experiment was done in duplicate. The C_t values in log linear range representing the detection threshold values were used for quantitation and expressed as copy numbers based on a standard curve generated using plasmid DNA.

Northern blot analysis. A multiple tissue Northern blot (MTN Blot II) containing poly-A (+) RNAs isolated from human adult tissues was obtained from Clontech. Northern hybridization was performed at 68°C in 10 ml of PerfectHybTM Plus hybridization solution (Sigma, St Louis, MO). The blot was pre-hybridized in the above buffer for 30 minutes. Hybridization was done for 1 hour by adding 50 ng of full length Sprouty1 or β -actin cDNA fragment that was radioactively labeled with [α -³²P] dCTP (3000 Ci/mmol; NEN Life Sciences) using a RadPrime

Labeling Kit (Invitrogen) and included at a concentration of 1×10^9 cpm/ml. Blots were washed according to the manufacturer's protocol and signals visualized by autoradiography.

Cell Culture. The human prostate cancer cell lines, PC3, DU145 and LNCaP, and the immortalized normal prostate epithelial cell lines PNT1a and PNT2 were maintained in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). NIH3T3 cells were maintained in high-glucose DMEM and 10% fetal bovine serum. Primary epithelial cultures were established from normal peripheral zone tissue from radical prostatectomy specimens as described previously ³³.

Stable transfections and selection. For stable transfections, PC3 or LNCaP cells were seeded at 5×10^6 cells per 100 mm dish and transfected with 10 μ g of Sprouty1 construct (pcDNA-Sprouty1) or vector only (pcDNA3.1) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. For stable transfections, 2 days after transfection, cells were selected in Geneticin (Sigma) containing medium at a final concentration of 400 μ g/ml and 200 μ g/ml for LNCaP and PC3 cells respectively. After fourteen days into the selection individual Geneticin resistant colonies were fixed with 10% formalin and stained with crystal violet, and the colonies visible to the naked eye counted.

Luciferase reporter assay. The prostate cell lines PC3, DU145 and LNCaP and mouse cell line NIH3T3 were plated at 3×10^5 cells per 60 mm dish and transiently transfected with 2 μ g of pGL-Sprouty1 and 2 μ g of β -galactosidase expression plasmid (pCH110, Pharmacia, Picataway, NJ) using 6 μ l of Fugene 6 transfection reagent (Invitrogen) according to the manufacturer's protocol. After 23 hours, cells were placed in growth medium containing either insulin as the only growth factor (1% ITS, Sigma) or insulin and 100 ng/ml FGF2. Cell lysates were collected after an additional 24 hour incubation. Luciferase activity was determined using a Luciferase

Assay kit (Roche) according to manufacturer's protocol after normalization for β -galactosidase activity using β -Gal Reporter Gene Assay Kit (Roche) according to the manufacturer's protocol.

FGF2 induction studies. Primary epithelial, LNCaP and PC3 cells were placed in serum free medium for 24 hours. Cells were refed with serum free medium with 1% ITS (Sigma) with or without 25ng/ml of recombinant FGF-2 (R&D Systems, Minneapolis, MN) and incubated at 37°C for different time points. Cells were then harvested, RNA extracted and used in real-time quantitative PCR as described above.

RESULTS

Expression of Sprouty1 in human prostate tissue and cell lines. Initial studies were done to investigate the expression of the human Sprouty1 homologue in adult human tissues. A multiple tissue Northern blot was hybridized to the full length Sprouty1 cDNA (Figure 1A). Results show a single transcript (of approximately 3.5 Kb) in all of the tissues analyzed except peripheral blood leukocytes with the strongest expression observed in the prostate and the testis. To evaluate Sprouty1 expression *in vitro*, total RNA samples derived from normal prostatic peripheral zone (PZ) and 2 immortalized but non-tumorigenic prostatic epithelial cell lines (PNT1a and PNT2) were analyzed for Sprouty1 expression by RT-PCR reaction. As can be seen in Figure 1B, Sprouty1 is easily detectable in all three RNAs. Thus Sprouty1 is expressed in human prostate *in vivo* and in prostate epithelial cells *in vitro*.

Decreased Sprouty1 expression in human prostate cancer tissues.

In order to determine whether Sprouty1 is down-regulated in human prostate cancers, we carried out quantitative real-time PCR ³⁴ analysis. We quantitatively analyzed the expression level of Sprouty1 in a total of 37 prostate RNA samples (9 normal peripheral zone (PZ); 5 benign prostate hyperplasia (BPH), 20 clinically localized cancer (consisting of at least 70% cancer) by

real-time PCR. We quantified β -actin as an endogenous mRNA control. The real-time data is presented as the ratios of Sprouty1 mRNA transcripts $\times 10^3$ / β -actin transcript for each group of samples analyzed (Figure 2A). The BPH samples had a slightly higher level of sprouty-1 expression than normal peripheral zone tissue. We have shown previously that FGF2 is present at higher concentrations in BPH tissue², and given that FGFs can induce Sprouty1 in other systems, this finding is not unexpected. In contrast, Sprouty1 expression was about 70% higher in normal prostate tissues (6.60 ± 2.0 , SEM) compared to prostate cancers (3.82 ± 1.1 , SEM), despite the fact that FGFs are significantly upregulated in the vast majority of prostate cancers. Of the 20 cancers analyzed, 16 had Sprouty1 mRNA levels lower than the mean Sprouty1 mRNA level in normal tissue. The difference in Sprouty1 mRNA level between normal and cancer tissue was statistically significant ($p < 0.035$, t test). Similar results were obtained when Sprouty1 expression was normalized using keratin 18 mRNA, which is expressed exclusively by epithelium (Figure 2B). Expression of Sprouty-1 normalized for epithelial content was almost 2 fold higher in normal tissues when compared to cancers (3.8 ± 0.9 Vs 2.0 ± 0.2) and this difference was again statistically significant ($p < 0.02$, t test).

One possible explanation for the decreased amounts of Sprouty1 mRNA in cancer tissues could be that Sprouty1 is expressed at higher levels in stroma compared to epithelium *in vivo* and that in cancer the stroma is replaced by neoplastic epithelium. If this were the case, one would expect that there would be an inverse correlation between the level of keratin 18 mRNA and Sprouty1 mRNA content in tissue samples. There was a 5-fold variation among the benign prostate tissues in keratin 18 mRNA content (relative to β -actin) due to variation in the percentage of epithelium in the tissue as a result of sampling variability. However, there was no correlation (inverse or positive) between keratin 18 mRNA levels and Sprouty1 mRNA levels.

This is consistent with a relatively equal expression of Sprouty1 mRNA in the normal epithelial and the stromal compartments *in vivo*. This is concordant with our finding that there is almost equal expression of Sprouty1 mRNA in primary epithelial and stromal cultures *in vitro* as determined by quantitative real-time PCR.

Effect of FGF2 on Sprouty1 expression in normal and neoplastic prostate epithelial cells.

To investigate whether Sprouty1 expression is regulated in response to fibroblast growth factor stimulation in the prostate epithelial cells, we examined the effect of FGF2 stimulation on Sprouty1 expression *in vitro*. Figure 3 shows that when primary (1^o) epithelial cells were stimulated with FGF2, there was a 5-fold increase in Sprouty1 expression within 30 minutes. However, this expression was rapidly down-regulated to below basal level in 1 hour. There was a subsequent increase in Sprouty1 expression after 2 hours that was sustained, peaking again within 24 hours, suggesting that there is a biphasic increase in expression of Sprouty1 in response to FGF2 stimulation. In contrast to 1^o epithelial cells, LNCaP and PC3 cells did not show induction of Sprouty1 expression in response to FGF2 stimulation. In fact, there was a slight down-regulation of Sprouty1 at 30 minutes or 2 hours after FGF stimulation in PC3 and LNCaP cells, respectively. This result indicates that, unlike normal prostate cells, Sprouty1 expression in prostatic cancer cells is no longer up-regulated by FGF2.

Absence of transcriptional regulation of the Sprouty1 promoter by FGF2 in prostate cancer cells. To determine if the inability of prostate cancer cells to increase Sprouty1 messenger RNA levels in response to FGF2 was due to changes in transcriptional regulation of the Sprouty1 gene, we carried out transient transfections in the absence or presence of FGF2 with a plasmid containing a reporter gene under the control of the Sprouty1 promoter. A typical experiment is shown in Figure 4. FGF2 increased transcription from the Sprouty1 promoter by

approximately 2.5 fold in NIH3T3 cells. In contrast, there was no significant increase in transcription from the Sprouty1 promoter in the prostate cancer cell lines in response to FGF2. These findings are consistent with the hypothesis that there is loss of transcriptional regulation of Sprouty1 in response to FGF stimulation in prostate cancer cells.

Mutational analysis of Sprouty1 in human prostate cancers. To determine if Sprouty1 is inactivated by mutation in prostate cancer, we analyzed DNAs isolated from 24 prostate cancers (23 clinically localized and one metastatic). Our initial analysis of the human genome database revealed that the entire coding region (and 3' untranslated region) is present on a single exon. We therefore designed PCR primers to amplify the entire coding region as overlapping PCR products that were then isolated and directly sequenced. All tumor specimens were at least 80% carcinoma and we have detected regions of loss of heterozygosity in all of these specimens using PCR-based approaches. A single bp alteration (T to C) was detected in one clinically localized prostate cancer at bp 1250 that would lead to an amino acid change from tyrosine to histidine at amino acid residue 304 of the Sprouty1 protein. Analysis of DNA from benign tissue from the same patient revealed the exact same alteration. Therefore, this sequence variation represents either a germline mutation or a relatively uncommon polymorphism. No evidence of mutation was seen in the Sprouty1 coding region in any other sample.

The effect of Sprouty1 expression in human prostate cancer cells. To ascertain the biological effect of Sprouty1 expression in human prostate cancer, pcDNA-Sprouty1 (encoding the full length of Sprouty1 sequence) was transfected into the human prostate cancer cell lines LNCaP and PC3 and transfected cells selected in Geneticin. Only rare colonies were observed in both the LNCaP and PC3 cells transfected with the Sprouty1 plasmid, whereas numerous colonies were observed when PC3 and LNCaP were transfected with the vector only plasmid (Figure 5). The

inhibition of colony formation by Sprouty1 was over 99%, suggesting that over-expression of Sprouty1 has a markedly deleterious effect on prostate cancer cells proliferation and/or survival.

DISCUSSION

One important way that cancers can grow in an uncontrolled way is by expressing increased amounts of growth factors and/or having increased activity of growth factor receptors. Cancers may also exhibit loss of regulatory factors that control the activity of growth factors receptors. For example, inactivation of the PTEN gene, which is a negative regulator of the phosphatidylinositol 3-kinase pathway, is inactivated in a variety of human malignancies, including prostate cancer³⁵. In the present study we have found that one protein that may have an important role in controlling growth signals, Sprouty1, is down-regulated in human prostate cancer tissues when compared to normal prostate tissue. In humans, there is up-regulation of FGFs in prostate cancer when compared with uninvolved prostate. Loss of Sprouty1 expression may give rise to unrestrained signal transduction by FGFs that could result in increased proliferation^{2,6} and/or decreased cell death³⁶ in prostate cancer and potentiate the effects of increased FGFs and FGF receptors in prostate cancer.

We have found that Sprouty1 expression in prostate cancer cells is no longer upregulated by FGF2. Furthermore, our studies investigating Sprouty1 promoter activity indicate that there is loss of transcriptional regulation of Sprouty1 in prostate cancer cells in response to FGFs. This could be due to alterations of trans-acting factors, such as loss of essential transcription factors or upregulation of negative regulatory factors which are yet to be determined. It should be noted that examination of the Cancer Genome Anatomy database reveals that Sprouty2, Sprouty3 and Sprouty 4 are all expressed in prostate, although at lower levels than Sprouty1, and all of these genes appear to be down-regulated in prostate cancer, suggesting the possibility of a common

mechanism leading to decreased transcription of the various forms of Sprouty in prostate cancer. Further work is needed in order to understand the molecular mechanisms that lead to decreased Sprouty1 transcription in prostate cancer.

The loss of expression of Sprouty1 in prostate cancer *in vivo* could also be due to alterations in the gene itself, such as deletion or methylation. The Sprouty1 gene maps to chromosome 4q27 (www.ncbi.nlm.nih.gov/LocusLink). Comparative genomic hybridization shows loss of this region in 23% of prostate cancers examined³⁷ so it is possible that in some cases decreased Sprouty1 expression could be due to hemi- or homozygous deletion of the Sprouty1 locus. Methylation has been shown to be involved in loss of gene expression in prostate cancer³⁸⁻⁴⁰. Systematic studies of Sprouty1 promoter methylation and correlation with gene expression in prostate cancers *in vivo* will need to be carried out to exclude this possibility. The relative importance of transcriptional regulation, deletion and methylation in control of Sprouty1 expression in prostate cancers *in vivo* will require further studies.

In summary, there is considerable evidence showing up-regulation of fibroblast growth factors in prostate cancer based on studies in animal models, human tissues and human prostate cancer cell lines. Sprouty1, an inhibitor of FGF signal transduction, is down-regulated in prostate cancer and may lead to the unrestrained signal transduction by FGFs and hence tumor progression. Currently we are examining whether loss of Sprouty1 expression is correlated with tumor progression and clinical outcome in human prostate cancer. Because Sprouty1 may inhibit the transduction of many growth factor signals, it could be an attractive target to explore for drug intervention or gene therapies of prostate cancer.

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Figure legends

Figure 1. Expression of Sprouty1 in the prostate and other human tissues. A. A multiple tissue Northern blot (MTN Blot II) containing poly-A (+) RNAs (Clontech) was hybridized to radiolabeled Sprouty 1 cDNA (upper panel). The blot was reprobed with radiolabeled β -actin cDNA (lower panel). The RNA samples analyzed are; S (Spleen), Th (Thymus), P (Prostate), T (Testis), O (Ovary), SI (Small Intestine), C (Colon; no mucosa) and PB (Peripheral blood leukocyte). B. RT-PCR analysis of RNAs from normal prostatic peripheral zone tissue (PZ) and two immortalized prostatic epithelial cell lines, PNT1a and PNT2.

Figure 2. Sprouty1 expression in normal and hyperplastic prostate and prostate cancer as determined by quantitative RT-PCR. Sprouty1 expression in normal prostatic peripheral zone (PZ), benign prostate hyperplasia (BPH) and cancer tissues was assessed by quantitative RT-PCR using a real-time thermal cycler (iCycler, BioRAD). Sprouty1 expression levels are displayed as a ratio of Sprouty1 transcripts $\times 10^3$ to β -actin transcripts (A) or Sprouty1 transcripts $\times 10^2$ to keratin 18 transcripts (B). The Sprouty1, β -actin and keratin 18 values were calculated from standard curves. The data is a representative of duplicate experiments. Values are the mean \pm SEM. The Sprouty1 expression value form cancer cases is significantly different from the PZ, $p<0.05$ (t test) for both β -actin and keratin 18 normalization.

Figure 3. Sprouty1 expression in response to FGF2 stimulation of normal or neoplastic prostate epithelial cells. Prostatic 1° epithelial cells, LNCaP and PC-3 cells were grown in serum free medium for 24 hours. Cells were stimulated with serum free medium with or without 25ng/ml of recombinant FGF2. At different time points the Sprouty1 expression in the cells were analyzed by quantitative RT-PCR using total RNA. Sprouty1 expression levels are displayed as the ratio of Sprouty1 to β -actin to correct for variation in the amounts of reverse-transcribed

RNA, with the ratio before FGF2 treatment set as 100% for each cell line. The Sprouty1 and β -actin values were calculated from Sprouty1 and β -actin standard curves respectively. The data is a representative of duplicate experiments. Values are the mean \pm SD.

Figure 4. Transcriptional activity of the Sprouty1 promoter by FGF2 in prostate cells.

NIH3T3, PC3, DU145 and LNCaP cell lines were transiently transfected with a luciferase reporter construct under the control of the Sprouty1 promoter. Transfected cells were treated with or without FGF2 and luciferase activity in cell lysates determined as described. The luciferase activity of Sprouty1 promoter is shown as % change in reporter activity compared to control (no FGF2 set as 100%) in each cell line.

Figure 5. Stable transfections of Sprouty1 wild-type plasmids into prostate cancer cells.

Prostate cancer cell lines LNCaP and PC3 were each transfected with a Sprouty1 cDNA cloned into pcDNA3.1 or the pcDNA3.1 vector alone. After 2 weeks of selection in Geneticin, cells were fixed and stained with crystal violet. Representative plates from each transfection is shown.

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Figure 1

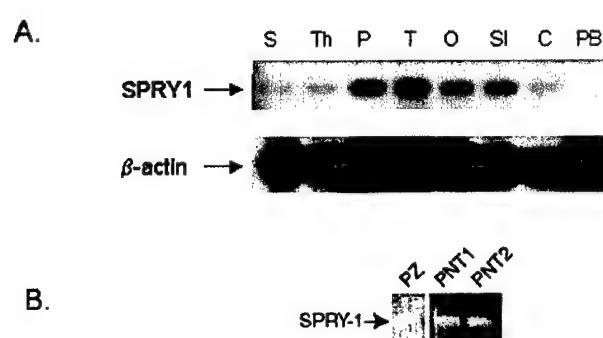


Figure 2

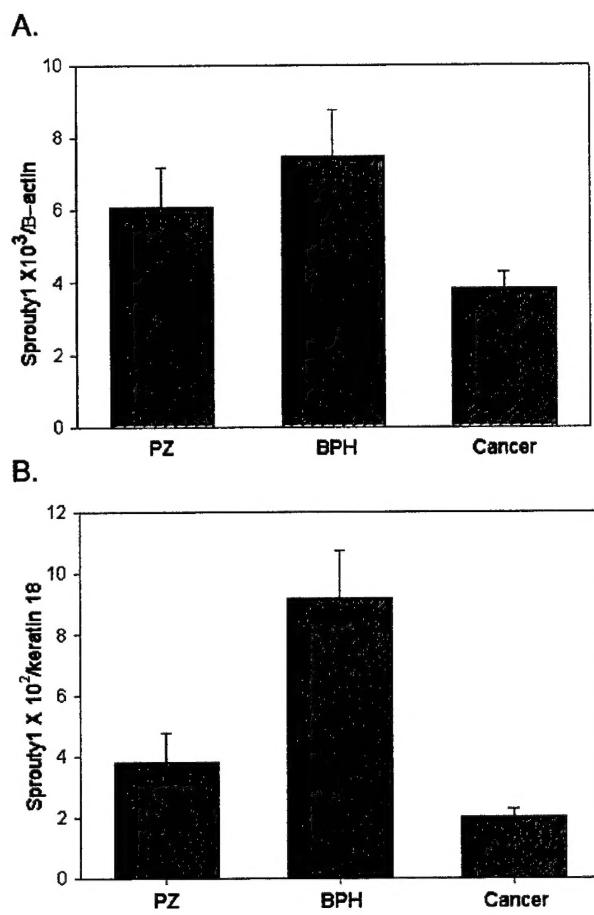


Figure 3

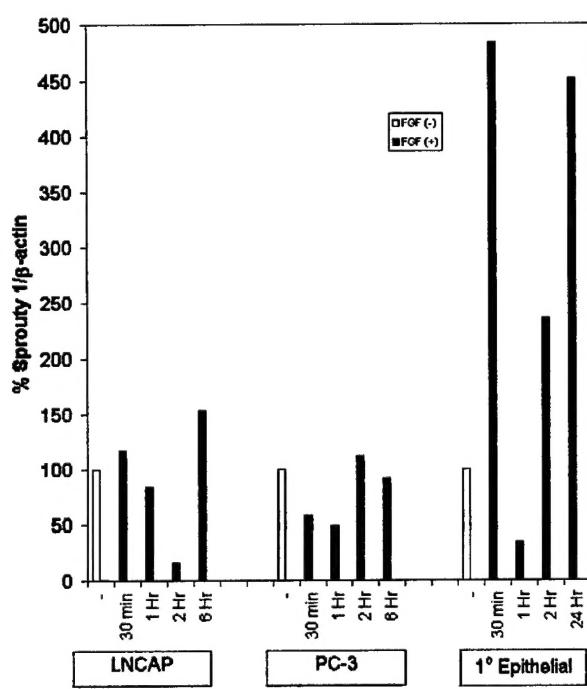


Figure 4

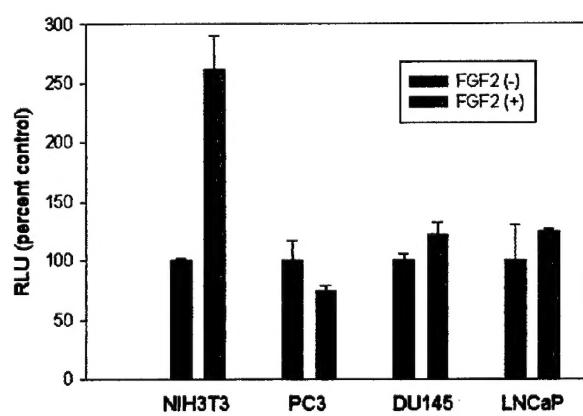


Figure 5

